Chapter 6

Plasma cholesteryl ester transfer is a determinant of intima-media thickness in Type 2 diabetic and non-diabetic subjects: role of CETP and triglycerides

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Abstract

Objective

We tested whether carotid artery intima-media thickness (IMT) is associated with plasma cholesteryl ester transfer (CET) and/or the plasma cholesteryl ester transfer protein (CETP) concentration in type 2 diabetic and control subjects.

Research Design and Methods

In 87 men and women with type 2 diabetes (non smokers; no insulin or lipid lowering drug treatment) and 82 control subjects, IMT, plasma CET, CETP mass and lipids were determined.

Results

HDL cholesterol was lower whereas IMT, pulse pressure, plasma triglycerides as well as plasma CET and CETP concentration were higher in diabetic patients *vs*. control subjects. In diabetic patients, plasma CET was positively determined by triglycerides ($p < 0.001$), non-HDL cholesterol ($p < 0.001$), CETP ($p = 0.002$) and by the interaction between CETP and triglycerides ($p = 0.004$). In control subjects, plasma CET was positively related to triglycerides ($p < 0.001$) and non-HDL cholesterol ($p < 0.001$). HDL cholesterol was inversely related to plasma CET in each group ($p < 0.01$ for both). IMT was positively associated with plasma CET in diabetic ($p = 0.05$) and control subjects ($p < 0.05$) after adjustment for age, gender and pulse pressure. No independent relationship with plasma CETP mass was found.

Conclusions

Plasma CET is a positive determinant of IMT. Plasma CETP mass, in turn, is a determinant of CET with an increasing effect at higher triglycerides. These data, therefore, provide a rationale to evaluate the effects of CETP inhibitor treatment on plasma CET and on cardiovascular risk in diabetes-associated hypertriglyceridemia.

Introduction

The inverse relationship between high density lipoprotein (HDL) cholesterol and cardiovascular disease is well documented in non-diabetic and type 2 diabetic populations [1,2]. Among other mechanisms, the cholesteryl ester transfer protein (CETP)-mediated process of plasma cholesteryl ester transfer plays a key role in HDL metabolism. CETP transfers cholesteryl esters from HDL towards lipoproteins of lower density classes in exchange for triglycerides [3-5]. Hence, the plasma cholesteryl ester transfer process is likely to contribute to low HDL cholesterol, as frequently observed in type 2 diabetes mellitus [5].

Only few studies have addressed the effect of plasma CETP levels and the rate of plasma cholesteryl ester transfer on cardiovascular risk in humans. A small study showed a positive correlation between the plasma CETP concentration and carotid artery intima-media thickness [6]. In addition, plasma CETP mass was shown to be a positive determinant of incident coronary artery disease but only in subjects with high triglycerides [7]. The cholesteryl ester transfer process itself could, however, also represent a beneficial pathway by enabling the transport of HDL-derived cholesteryl esters back to the liver via very low and low density lipoproteins (VLDL and LDL) [3]. The impact of the plasma cholesteryl ester transfer process on atherosclerosis development remains, therefore, uncertain [8].

CETP inhibitors, represent a new class of drugs that effectively raise HDL cholesterol [9,10]. While pharmacological inhibition of circulating CETP has been shown to retard atherosclerosis in animal experiments [11], the effect of this treatment on cardiovascular disease in humans is not yet known, nor is it clear which patient categories would benefit most from such treatment. Many studies have shown that plasma cholesteryl ester transfer is enhanced in type 2 diabetes mellitus [12-17]. This abnormality is largely ascribed to alterations in the concentration and composition of triglyceride-rich lipoproteins that accept cholesteryl esters from HDL, although the extent to which variations in the CETP concentration per se affect the rate of plasma cholesteryl ester transfer is still uncertain [5].

In view of a potentially deleterious effect of elevated plasma CETP levels on cardiovascular disease in hypertriglyceridemic subjects [7], we hypothesized that an increased cholesteryl ester transfer is a determinant of subclinical atherosclerosis in type 2 diabetes mellitus. In the present study we determined the contribution of the plasma CETP concentration to cholesteryl ester transfer, and established whether intima-media thickness (IMT) is associated with plasma cholesteryl ester transfer or the CETP concentration in type 2 diabetes mellitus.

Research design and methods

The study protocol was approved by the medical ethics committee of the University Medical Center Groningen and written informed consent was obtained from each participant. Type 2 diabetic patients and age-matched non-diabetic control subjects, aged > 18 years, were recruited by advertisement in local newspapers. Current or previous smoking or use of lipid lowering drugs were exclusion criteria since these factors would have introduced bias with respect to the evaluation of determinants affecting IMT. Subjects with micro- or macroalbuminuria defined as urinary albumin > 20 mg/l were also excluded. Maximal alcohol intake was 3 beverages per day.

Fifty-three male and 34 female type 2 diabetic patients were included in this study. The non-diabetic control group comprised 45 men and 37 women. Type 2 diabetes mellitus was previously diagnosed using blood glucose cut-off values as defined by the WHO, and patients were treated with diet alone (26%) or in combination with oral glucose-lowering agents (74%). Insulin treatment was an exclusion criterion. Oral glucose-lowering drugs used were sulfonylurea (33%), biguanides (26%) or the combination of these two (41%). Next to these drugs, 8 patients used a thiazolidinedione and 2 patients used acarbose. Forty-six percent of diabetic patients but none of the control subjects used one or more anti-hypertensive drugs (73% angiotensin converting enzyme inhibitors or angiotensin-II-receptor antagonists, 42% beta-blockers, 40% diuretics, 15% calcium antagonists). Seventy-nine percent of the female type 2 diabetic patients and 65% of the non-diabetic women were post-menopausal ($p = 0.18$). Fourteen percent of non-diabetic and 14 % of diabetic pre-menopausal women used oral contraceptives.

All participants were evaluated after an overnight fast. BMI was calculated as weight divided by height squared. Waist circumference was measured as the smallest circumference between rib cage and iliac crest. Systolic and diastolic blood pressure were measured, after at least 15 min of rest, at the left arm in sitting position using a sphygmomanometer. Pulse pressure was calculated as the difference between systolic and diastolic blood pressure.

Carotid IMT measurement

IMT of the carotid arteries was measured by ultrasonography in the supine position. High-resolution B-mode ultrasound images (ACUSON 128 XP, Mountain View, CA, USA) with a 7.0 MHz linear array transducer were used to measure intima-media wall thickness. The right and left common carotid arterial wall segments were imaged from a fixed lateral transducer angle at the far wall of the distal one centimetre segments of both common carotid arteries, proximal to the carotid bulb. The scans

were recorded on S-VHS tape and analyzed off-line by an independent image analyst unaware of subject characteristics. B-mode image analyses were digitized with a frame grabber (DT286 l; Data Translation Inc.; Marlboro, MA). The image analysis software was developed using an algorithm as developed by Selzer et al [18]. The mean IMT over the six segments of both carotid arteries was calculated and designated mean IMT. At a mean IMT of 0.80 mm, inter-sonographer variability amounted to 0.05 mm, with an image analyst variability of less than 0.03 mm, corresponding to a total variation coefficient between 6.3% and 7.3%.

Laboratory measurements

Venous blood samples for measurement of (apo)lipoproteins, cholesteryl ester transfer and CETP mass were collected into ethylene diamine-tetra-acetic acid (EDTA)-containing tubes (1.5 mg/ml) which were placed on ice immediately. Plasma was obtained within 30 min by centrifugation at 4˚C. Samples were kept frozen at -80˚C until analysis.

Plasma total cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat nos 11876023 and 11875540 respectively, Roche Diagnostics GmbH, Mannheim, Germany). HDL cholesterol was determined in the supernatant fraction after precipitation of apolipoprotein (apo) B-containing lipoproteins with polyethylene glycol-6000. Non-HDL cholesterol was calculated by subtracting HDL cholesterol from plasma total cholesterol. Apo A-I and apo B were measured by immunoturbidimetry (Roche /Cobas Integra Tina-quant cat nos 03032566 and 03032574 respectively, Roche Diagnostics GmbH, Mannheim, Germany).

Plasma cholesteryl ester transfer (CET), was assayed essentially as described previously [19,20]. In brief, [^{3}H]cholesterol was equilibrated for 24 h with plasma cholesterol at 4˚C followed by incubation at 37˚C for 3 h. Subsequently, apo B-containing lipoproteins were precipitated by addition of phosphotungstate/$MgCl_2$. Lipids were extracted from the precipitate and the labeled cholesteryl esters were separated from labeled unesterified cholesterol on silica columns.

Plasma CETP concentration was analyzed using a double-antibody sandwich ELISA [21]. A combination of monoclonal antibodies TP1 and TP2 was employed as coating antibodies and monoclonal antibody TP20, labeled with digoxigenine, as the secondary antibody. The CETP control samples were validated using a radioimmunoassay (carried out by Dr. R.M. McPherson, Montreal, Canada).

Glucose was analyzed with an APEC glucose analyzer (APEC Inc., Danvers, MA). Glycated hemoglobin was measured by high performance liquid chromatography (Bio-Rad, Veenendaal, The Netherlands; normal range 4.6-6.1%).

Statistical analysis

Data are shown as mean ± SD. In case of a skewed distribution, data are presented as median (interquartile range). Data were compared using unpaired t-tests. When data were not normally distributed, Mann-Whitney U tests were used. Chi-square analysis was used to evaluate differences in proportions of parameters. Multiple linear regression analysis was used to reveal independent relationships between variables. When variables had a skewed distribution, logarithmically transformed values were used in the models. For continuous variables, we subtracted the mean value from the measured value to obtain a distribution centered on the mean. In further models, we considered interactions between levels of the variables of interest by including product terms in the models. Two-sided p-values ≤ 0.05 were considered to be statistically significant.

Results

Table 1 summarizes clinical characteristics, carotid IMT measurement and glycemic control in type 2 diabetic patients and control subjects. Gender distribution ($p = 0.43$) and age were not different between the groups. Carotid IMT was higher in diabetic patients than in control subjects. In both groups IMT was higher in men compared to women. BMI and waist circumference were greater in diabetic patients than in control subjects, whereas diabetic women were more obese than diabetic men. Systolic and diastolic blood pressure as well as pulse pressure were higher in diabetic patients. Fasting glycemia and HbA1c levels were also higher in diabetic patients.

As shown in Table 2, plasma total cholesterol was moderately lower in type 2 diabetic patients compared to control subjects but non-HDL cholesterol and plasma apo B levels were not different between the groups. Plasma total cholesterol was slightly lower in diabetic men than in diabetic women. In diabetic patients, plasma triglycerides were higher, whereas HDL cholesterol and plasma apo A-I levels were lower compared to control subjects. In both the diabetic and the control group, HDL cholesterol and plasma apo A-I levels were higher in women than in men. Plasma CET as well as CETP concentration was higher in type 2 diabetic patients compared to control subjects. Among diabetic patients plasma CETP was higher in women than in men but the difference in CET was not significant. There was no significant effect of menopausal status on plasma CETP in either group (data not shown; $p > 0.10$ for both).

Table 1. Clinical characteristics and mean carotid artery intima-media thickness (IMT) in type 2 diabetic patients and control subjects

	Type 2 diabetic patients N = 87		*Control subjects N = 82*		
	Men	Women	Men	Women	P-value
N	53	34	45	37	0.43
Age (years)	59 ± 9	56 ± 8	58 ± 10	53 ± 7*	0.15
Carotid IMT (mm)	0.91 ± 0.19	0.81 ± 0.20*	0.86 ± 0.15	0.77 ± 0.12*	0.025
BMI (kg/m^2)	27.9 ± 4.4	30.7 ± 5.6*	26.1 ± 3.3	25.1 ± 3.9	<0.001
Waist circumference (cm)	102 ± 13	99 ± 14	94 ± 10	81 ± 12†	<0.001
Systolic blood pressure (mmHg)	141 ± 18	146 ± 20	132 ± 16	132 ± 21	<0.001
Diastolic blood pressure (mmHg)	86 ± 9	88 ± 8	84 ± 9	81 ± 12	0.003
Pulse pressure (mmHg)	55 ± 16	58 ± 18	48 ± 11	51 ± 17	0.005
Glucose (mmol/l)	8.4 ± 1.8	9.4 ± 2.7	5.8 ± 0.7	5.5 ± 0.7*	<0.001
HbA1c (%)	6.7 ± 0.9	6.8 ± 1.1	5.4 ± 0.4	5.3 ± 0.4	<0.001

Data in mean ± SD. P-values for the difference between type 2 diabetic patients (men and women combined) and control subjects (men and women combined) are shown. Within group difference between men and women: *p < 0.05; †p < 0.001

Table 2. Plasma lipid parameters, cholesteryl ester transfer (CET) and cholesteryl ester transfer protein (CETP) concentration in type 2 diabetic patients and control subjects

	Type 2 diabetic patients N = 87		*Control subjects N = 82*		
	Men	Women	Men	Women	P-value
N	53	34	45	37	0.43
Plasma total cholesterol (mmol/l)	5.2 ± 0.9	5.6 ± 0.8*	5.6 ± 1.0	5.8 ± 0.8	0.008
Non-HDL cholesterol (mmol)	3.87 ± 0.96	4.16 ± 0.90	4.16 ± 1.03	3.98 ± 0.79	0.51
HDL cholesterol (mmol/l)	1.28 ± 0.33	1.46 ± 0.37*	1.49 ± 0.36	1.82 ± 0.41†	<0.001
Plasma triglycerides (mmol/l)	1.82 (0.97 –2.46)	1.64 (1.26 – 2.15)	1.34 (0.94 – 2.07)	1.21 (0.86 – 1.73)	0.008
Plasma apo A-I (g/l)	1.26 ± 0.17	1.43 ± 0.28†	1.36 ± 0.21	1.53 ± 0.22†	0.001
Plasma apo B (g/l)	0.90 ± 0.20	0.96 ± 0.22	0.99 ± 0.26	0.90 ± 0.19	0.49
Plasma CET (nmol.ml^{-1}.h^{-1})	23.3 ± 8.1	25.0 ± 9.5	20.6 ± 7.3	19.7 ± 4.7	0.001
Plasma CETP concentration (mg/l)	2.36 ± 0.83	2.90 ± 0.99*	2.16 ± 0.68	2.17 ± 0.65	0.001

Data in mean ± SD or in median (interquartile range). P-values for the difference between type 2 diabetic patients (men and women combined) and control subjects (men and women combined) are shown. Within group difference between men and women: *p < 0.05; †p < 0.001

Multiple linear regression analyses showed that in type 2 diabetic patients plasma CET was independently and positively determined by plasma triglycerides (log transformed, $p < 0.001$), non-HDL cholesterol ($p < 0.001$), plasma CETP ($p = 0.002$) and by the interaction between plasma CETP and triglycerides ($p = 0.004$, multiple $r = 0.84$) (Fig. 1). There was no effect of antihypertensive medication on plasma CET ($p = 0.75$). Multiple linear regression analysis showed that in diabetic patients plasma CETP concentration was higher in women ($p = 0.004$) but was unrelated to BMI ($p = 0.24$) and the use of anti-hypertensive drugs ($p = 0.83$). In control subjects, plasma triglycerides ($p < 0.001$) and non-HDL cholesterol ($p < 0.001$), but not plasma CETP mass ($p = 0.20$), were independent determinants of plasma CET (multiple $r = 0.75$). In each group, HDL cholesterol was negatively associated with plasma CET ($p < 0.001$ and $p = 0.007$ in diabetic and control subjects, respectively) and positively with female gender ($p = 0.003$ and $p < 0.001$; multiple $r = 0.52$ and 0.48 in diabetic and control subjects, respectively).

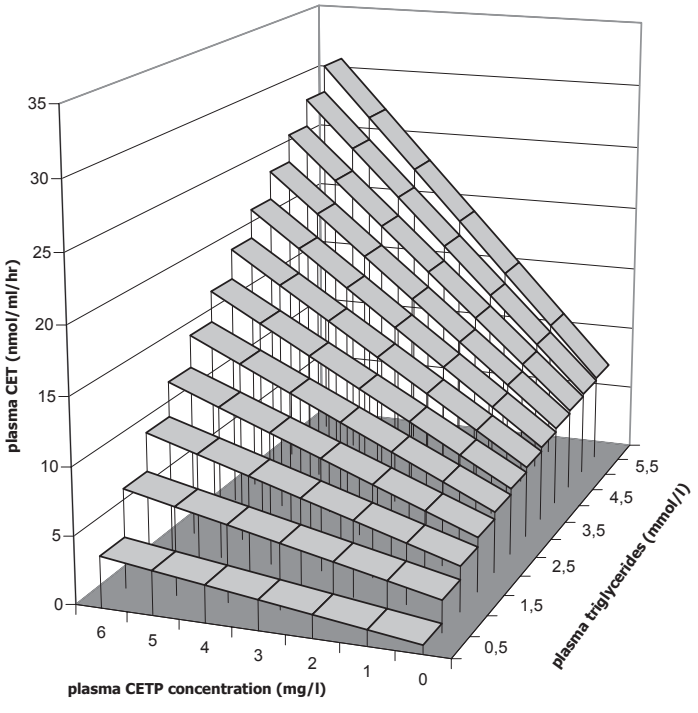

Figure 1. Graphical presentation of the interaction between the plasma cholesteryl ester transfer protein (CETP) concentration and plasma triglycerides on plasma cholesteryl ester transfer (CET) in type 2 diabetic patients

Table 3. Determinants of carotid artery intima-media thickness (IMT) in 87 type 2 diabetic patients and 82 control subjects by multiple linear regression analysis

a: Type 2 diabetic patients

	B	*P*
Constant	$4.55 . 10^{-2}$	0.74
Age (years)	$8.43 . 10^{-3}$	<0.001
Gender (M/F)	$9.10 . 10^{-2}$	<0.05
Pulse pressure (mmHg)	$3.34 . 10^{-3}$	<0.01
Plasma CET (nmol/ml/h)	$4.13 . 10^{-3}$	0.05

b: Control subjects

	B	*P*
Constant	$21.4 . 10^{-2}$	0.02
Age (years)	$5.89 . 10^{-3}$	<0.001
Gender (M/F)	$6.13 . 10^{-2}$	<0.05
Pulse pressure (mmHg)	$3.18 . 10^{-3}$	0.001
Plasma CET (nmol/ml/h)	$4.02 . 10^{-3}$	<0.05

CET: cholesteryl ester transfer

Multiple linear regression analyses were also carried out to determine whether IMT was associated with plasma CET and/or the CETP concentration. In these analyses age, gender and pulse pressure (as best fitting hemodynamic variable) were included since these variables are recognized to be strong determinants of IMT [22,23]. In each group, plasma CET was a positive determinant of IMT after adjustment for age, gender and pulse pressure (Table 3; multiple r = 0.60 and 0.48 in diabetic and control subjects, respectively). IMT was not independently associated with plasma CETP mass in diabetic patients (p = 0.84) and control subjects (p = 0.89).

Discussion

We have demonstrated for the first time that plasma cholesteryl ester transfer is a determinant of IMT in type 2 diabetic patients and non-diabetic control subjects. These findings support the hypothesis that a high transfer of cholesteryl esters from HDL to apo B-containing lipoproteins is involved in the development of atherosclerosis. Plasma cholesteryl ester transfer in type 2 diabetic patients was elevated compared to control subjects as expected [12-17], and in each group HDL cholesterol was inversely related to plasma cholesteryl ester transfer. Besides positive effects of the plasma triglyceride

level and non-HDL cholesterol, the plasma CETP concentration per se contributed to plasma cholesteryl ester transfer in diabetic patients. Furthermore, the effect of the plasma CETP level on plasma cholesteryl ester transfer becomes more important with higher plasma triglycerides. Our data, therefore, agree with the concept that manoeuvres aimed at inhibiting active CETP mass in plasma may attenuate the cholesteryl ester transfer process in diabetes-associated hypertriglyceridemia, which in turn could favorably affect cardiovascular risk.

The isotopic procedure employed to determine plasma cholesteryl ester transfer in the present study represents an accurate measure of net cholesteryl ester mass transfer from HDL to apo B-containing lipoproteins [13]. Abnormalities in the concentration and composition of apo B-containing lipoproteins that accept cholesteryl esters from HDL are largely responsible for the increased plasma cholesteryl ester transfer in type 2 diabetes mellitus [12,17]. In the current study, we found that the plasma triglyceride and non-HDL cholesterol levels contribute to plasma cholesteryl ester transfer in diabetic and non-diabetic control subjects. As expected plasma triglycerides were higher in diabetic patients but non-HDL cholesterol was not different between the groups. This could be due to selection of participants since criteria for lipid lowering treatment are stricter for diabetic patients, and we intentionally only included subjects who never used lipid lowering drugs. Consequently, diabetic patients with relatively normal plasma cholesterol levels may have been preferentially included. We have also demonstrated that the plasma CETP concentration itself affects cholesteryl ester transfer independent from triglycerides and non-HDL cholesterol. This suggests that the CETP concentration is a determinant of plasma cholesteryl ester transfer at any plasma level of triglycerides and non-HDL cholesterol concentration, and agrees with our previous findings in a much smaller group of patients [16]. A potentially important observation is the positive interaction between plasma CETP mass and triglycerides on plasma cholesteryl ester transfer, which underscores that the contribution of the plasma CETP concentration as such to cholesteryl ester transfer becomes more important with higher plasma triglycerides. In comparison, a previous *in vitro* study has shown that variation in the plasma CETP level only affects net cholesteryl ester mass transfer from HDL to VLDL at high plasma triglyceride concentrations [24]. Another study demonstrated a negative correlation of HDL cholesterol with the plasma level of active CETP in hypertriglyceridemic men [25].

The plasma CETP concentration was found to be higher in the diabetic group. With few exceptions [26,27] most previous studies found unaltered plasma CETP activity levels per se in type 2 diabetes mellitus [16,17,28,29]. Plasma CETP activity, determined using an assay that measures the amount of active CETP, is closely correlated

with its mass in non-diabetic as well as in diabetic patients [30]. However, there are differences in the relationship between plasma CETP mass and its activity in diabetic compared to healthy subjects, as evidenced by a lower ratio of CETP activity divided by its mass in diabetes mellitus [30]. This may, at least, in part explain the discrepancy with previous plasma CETP activity data. The effect of the diabetic state on plasma CETP concentration appeared to be attributable to an increased level in diabetic women. The effect of obesity on plasma CETP mass may be absent in diabetes [31], and plasma CETP was unrelated to BMI in the presently studied diabetic patients. Therefore, the higher BMI in diabetic women compared to men does not explain their higher plasma CETP concentration. Since other reports also showed slightly higher plasma CETP levels in women [32,33], a gender effect as such is possible. However in the diabetic group, this gender effect on plasma CETP did not result in a significant difference in plasma cholesteryl ester transfer between men and women. This is probably explained by the slightly lower plasma triglyceride levels in diabetic women.

Carotid artery IMT, as measured by ultrasonography, predicts the development of cardiovascular disease [34]. It is unequivocally established that IMT is determined by age, gender and hemodynamic factors [22,23], which was again documented in the present study. Previous reports have shown that IMT may also be associated with non-HDL cholesterol, HDL cholesterol and plasma triglycerides [35,36]. It should be noted that we did not attempt to establish whether the relationship of IMT with plasma cholesteryl ester transfer was independent from these lipoprotein and lipid levels, because there were strong interrelationships between plasma apo B-containing lipoproteins, cholesteryl ester transfer and HDL cholesterol. It was recently documented that the plasma CETP concentration determines cardiovascular risk in hypertriglyceridemic subjects [7]. Our study did not demonstrate an independent relationship of plasma CETP with IMT, neither in diabetic nor in control subjects. In view of present results the possibility is raised that an enhanced plasma cholesteryl ester transfer due to elevated plasma triglycerides in conjunction with high CETP is associated with cardiovascular disease rather than an elevated CETP level alone.

Limitations of our study include its observational nature and the restricted number of diabetic patients which only used oral hypoglycemic drugs. Metabolic control was adequate in most diabetic patients. Since glycation of CETP and of HDL-related apolipoproteins may influence the cholesteryl ester transfer process, further data are needed to establish the association of plasma cholesteryl ester transfer with (subclinical) atherosclerosis in poorly controlled diabetes [37].

It is obvious that the effect of CETP inhibitor treatment on cardiovascular disease can only be tested in clinical end-point studies. Furthermore, it is important

to realize that therapeutic measures aimed at lowering plasma cholesteryl ester transfer are not restricted to drugs that inhibit CETP activity. Both fibrate and statin therapy reduce plasma cholesteryl ester transfer by decreasing cholesteryl acceptor lipoproteins, i.e. VLDL and LDL [5,21]. Moreover, statins additionally lower the plasma CETP level [5,21,38]. Keeping this in mind, the present data provide a metabolic background in support of the hypothesis that lowering of plasma CETP may ameliorate cardiovascular risk in diabetes-associated hypertriglyceridemia.

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